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TEMPERATURE AND END-PLATE CURRENTS IN RAT DIAPHRAGM

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SUMMARY

1. Spontaneous miniature end-plate currents (m.e.p.c.s) were recorded in rat diaphragm at 7, 22 and 37 °C at −80 mV. The onset rate, measured as 20–80 % rise time, was sensitive to temperature with activation energy 14 kcal mol^{-1} deg^{-1}, and was not sensitive to membrane voltage between −60 and −130 mV.

2. The rise time recorded by external electrodes was 144 μs at 37 °C (6) and was similar to that found by internal electrodes.

3. The fall time was temperature-sensitive with activation 18 kcal, and was prolonged when the end-plate was hyperpolarized.

4. With acetylcholine (10 μM) the current increased to a peak and then fell within 30 s to a value which declined slowly. From fluctuation analysis the channel open time of 237 μs (7) at 37 °C was estimated. External recording gave comparable values (4). Comparison of the initial estimates with those obtained after 3–6 min of continued application showed no consistent change. The channel conductance was 26 pS at 37 °C.

5. The time constant of m.e.p.c. decay was consistently longer than the channel open time obtained from noise analysis.

6. With carbachol (40 μM) the current increased to a peak and then declined to a steady value. Fluctuation analysis by internal and external recording gave an increase of 5 % in root mean square current with channel open time of 83 μs (6) at 37 °C, and channel conductance 17 pS.

INTRODUCTION

The transmitter acetylcholine is likely to act on the post-synaptic area of the muscle end-plate at saturating concentrations (Matthews-Bellinger & Salpeter, 1978). The rising phase of a miniature end-plate current produced by a single quantum of transmitter may be attributed to the time required for release of transmitter, diffusion in the synaptic cleft, binding and activation of the receptor–channel complex (Land, Salpeter & Salpeter, 1980), and if diffusion is a major factor the activation energy of the rising phase should be low. Gage & McBurney (1975) reported in toad muscle that the rise time is relatively insensitive to temperature with a Q_{10} of 1·23. However, Datyner & Gage (1980) found that lowering the temperature prolonged markedly the onset time in mouse muscle, and Dwyer (1981) has recorded a high temperature coefficient for the rising phase in frogs. In the present study the

rising phase of the spontaneous miniature end-plate currents has been measured in rat muscle at temperatures between 7 and 37 °C. The design has also allowed the effect of voltage change on the rise and decay to be measured, and comparison has been made between decay rate and the channel open time estimated from noise analysis. At 37 °C with internal recording and high-resistance electrodes the clamp circuit can impose limitations on the analysis of high-frequency events, and for this reason external recordings were also made in conditions in which the clamp circuit was not involved.

METHODS

Preparation. A section of diaphragm, 5 mm wide and continuous from rib to tendon, was rapidly removed from rats killed by stunning, and placed in a recording chamber of volume about 2 ml through which solution flowed continuously at a rate between 40 and 45 ml min^{-1} at 37 and 22 °C, and 20 ml. min^{-1} at 7 °C. The muscle was stretched horizontally and held by pins in the Sylgard base. Loose connective tissue was removed by forceps and fine scissors.

Solutions. The solution was that of Krebs & Henseleit (1932) with modified potassium concentration so that the composition was (mM): Na^+, 145·2; K^+, 4; Mg^{2+}, 1·2; Ca^{2+}, 2·5; Cl^-, 128; HCO_3^-, 25; SO_4^{2-}, 1·2; $H_2PO_4^-$, 1·2; glucose, 11·1. The solution was gassed continuously with a mixture of 95% (v/v) oxygen and 5% (v/v) carbon dioxide, and the pH was 7·4 at room temperature after gassing for 1 h. The solution was held in a reservoir formed by a separating funnel and was cooled or heated as required by a concentric counter-current flow system on its route to the recording chamber. Two or more reservoirs were commonly used and solutions were switched by means of a tap. The time for arrival of fresh solution to the recording chamber was around 15 s at a flow rate of 40–45 ml min^{-1} and was dependent on flow rate; the attainment of a steady concentration in the chamber was assessed by dye studies and required an additional 10 s.

In some experiments two parallel counter-flow systems were maintained at different temperatures with a switching system which also changed the flow through the jacket of the recording chamber. By this method changes of temperature in the bathing solution could be achieved, as recorded by a thermistor probe in the recording chamber.

When acetylcholine was used, tetrodotoxin (300 nM) and physostigmine (3 μM) were also added.

Micro-electrodes. All micro-electrodes contained 3 M-potassium chloride. Intracellular voltage-recording electrodes had resistances of 15–30 MΩ; extracellular recording and current-passing electrodes had resistances of 8–15 MΩ. Electrodes were selected for low tip potential and low noise. Potential was recorded by a conventional differential cathode follower (two Mullard ME1400 electrometer valves) connected in parallel to two amplifiers of an oscilloscope, each with input resistance of 1 MΩ, and, via another cathode follower with low gain, to a digital voltmeter for direct potential read-out. One channel of the oscilloscope was d.c.-coupled with low gain for recording potential, the other channel was a.c.-coupled with higher gain for viewing miniature end-plate potentials (m.e.p.p.s).

The output of the cathode follower on occasion also fed a high-gain clamp amplifier (±18 V swing) used at a gain of 1000–1200 for passing current through an intracellular electrode. The current produced was assessed by another differential cathode follower as the voltage drop across a resistance of 1 MΩ in series with the current-passing electrode, and the output was connected to the two amplifiers of a second oscilloscope; one was a.c.-coupled with high gain to record miniature end-plate current (m.e.p.c.) and current noise (10 or 30 kHz low-pass filter, Gaussian response, −9 db per octave) and the other was connected to a d.c.-coupled low-gain amplifer for gross current measurement. Amplified outputs from the oscilloscope were recorded on an FM Store 4 Racal thermionic tape-recorder usually at speed of 60 in. s^{-1} (20 kHz low-pass, Bessel response, −24 db per octave) when looking at m.e.p.c. or 15 in. s^{-1} (5 kHz Bessel) or 30 in. s^{-1} (10 kHz Bessel) when looking at acetylcholine or carbachol responses. The effect of these filters upon a step response was to lengthen the 20–80% rise time to 18 μs for the 20 kHz Bessel filter on the tape-recorder and 24 μs for the 10 kHz Gaussian filter on the oscilloscope. At 37 °C the 20–80% rise time of m.e.p.c is likely to be lengthened, as a consequence of the frequency limitations of the circuitry, by up to 20% when the 10 kHz filter was used on the oscilloscope and by about 14% when the 30 kHz filter on the oscilloscope was used together with the 20 kHz filter in the tape-recorder. At lower temperatures

the 10 kHz filter was used and at 22 °C the rise of m.e.p.c. is unlikely to be lengthened by more than about 5% by these filter characteristics.

The preparation was viewed with a stereo-microscope at magnifications up to 100 times. The end-plate region was located by finding a position along the fibre where m.e.p.p.s with rise times of 400 μs or faster could be obtained. When clamping, a second electrode used to pass current was inserted into the cell less than 60 μm away from the recording electrode (this was less than 0·1 space constant: see Palade & Barchi, 1977). Pulses of 10 nA and 25 μs duration were passed to aid this impalement. Input resistance of each cell was noted to be in the range 0·1–0·9 MΩ. The clamping potential was set to −80 mV and the gain of the clamp amplifier was increased until the recorded potential was at the desired clamp value. With the cell clamped the spontaneous m.e.p.p. produced only a small deflexion on the voltage trace – usually less than 5% of the unclamped amplitude – and the current trace showed a fast onset and decay of current which was faster than an unclamped m.e.p.p. The end-plate region of rat diaphragm extends over a much shorter length of fibre (about 19–28 μm) than that in frog muscle (Cole, 1957) and it appeared that a cell was either adequately clamped or it was not; it did not seem that from one cell some m.e.p.c.s were better clamped than others (contrast the situation in frog muscle: Gage & McBurney, 1972). Although the initial membrane potential was usually more negative than −80 mV at 37 °C, or −74 mV at lower temperatures, it frequently decayed to a new level when two electrodes were inserted into the cell and this often – though not always – required the passage of current to clamp the potential at the holding potential of −80 mV. Recordings were considered acceptable only if the magnitude of the clamp current under the holding conditions did not exceed 15 nA and was steady.

If external recordings of inverted m.e.p.p.s were to be made the recording electrode was removed from the fibre and used to probe along either of its edges until inverted m.e.p.p.s could be recorded (Liley, 1956).

Analysis of drug-induced noise. The recorded signal was digitized at various rates: 1 kHz for acetylcholine (ACh) noise at 20 °C, 4 kHz for ACh noise at 37 °C, and 16 kHz for carbachol noise at 37 °C. When digitizing at 1 kHz the signal was passed through a 470 Hz low-pass filter (−3 dB at 470 Hz, 8 pole Butterworth active filter), and multiples of this were used when digitizing at the faster rates. The trace was viewed and blocks of data with m.e.p.c. or obvious base line changes were eliminated from the analysis. The length of the basic data block was different at the different digitization rates: 256 or 512 points ($\frac{1}{4}$ or $\frac{1}{2}$ s duration) at 1 kHz, $\frac{1}{8}$ s block duration at 4 kHz, and 512 or 1024 points at 8 kHz. The signal variance of the raw data was calculated, as was the one-sided spectral density function of these blocks after cosine tapering (Bendat & Piersol, 1971), the frequency resolution being 4, 8 or 16 Hz according to the specific case. The variance computed from the spectrum was adjusted to make allowance for the loss of signal by the tapering.

At 20 °C, fifty or a hundred blocks of data obtained during ACh application were analysed to produce an average spectrum and from this was subtracted an average spectrum from 100 blocks of data obtained before the addition of ACh. The resultant spectrum was plotted as log power against log frequency. At 37 °C 200 or 300 blocks were used both for control and ACh-induced results, and 400 blocks were used when carbachol was applied. A single Lorentzian curve, $S(f) = S(0)/[1+(f/f_c)^2]$, where $S(f)$ is the power spectral density at frequency f, f_c is half-power frequency and $S(0)$ is the spectral density at zero frequency, was fitted to the points by sliding a plastic template in the shape of the curve over the points. The $S(0)$ value was estimated as the median of points at low frequency, and the number of points to be included was obtained by a reiteration process until the inclusion of additional points produced a fall in the estimated $S(0)$ below the 95% limit; the $S(0)$ value and its confidence limits (Colquhoun, 1971) were then printed by the computer. In practice the time constant was estimated both by the relation $var = S(0)/4\tau$ (Wray, 1981), where *var* is the variance, and from the Lorentzian curve obtained by the template method, in which the value for $S(0)$ was constrained to be within the 95% limits which had been calculated; then the time constant was obtained from the relation $= 1/(2\pi f_c)$. When no plateau was obtained at low frequencies, the recordings were rejected.

Analysis of miniature end-plate currents (m.e.p.c.s) and inverted potentials (m.e.p.p.s). The m.e.p.c.s and m.e.p.p.s were analysed individually for their 20–80% rise time, rate of rise (nA/100 μs), amplitude, and time constant of decay, and means were obtained from at least ten m.e.p.c.s from one end-plate. An averaged m.e.p.c. (or inverted m.e.p.p.) was also calculated by scaling about the peak value and then recalculating the parameters. The values obtained by these two methods differed by less than 10%. Recordings were made with filters at 10 or 20 kHz as previously described,

and the signals were digitized at 32 kHz for a maximum duration of 16 s (point-to-point interval 31 μs). Blocks of data of at least 1280 points were searched by the computer which recognized the presence of m.e.p.c.; if a m.e.p.c. occurred within the first or last 256 points the signal was re-read with a change of origin to place the m.e.p.c. in the middle 768 points, so that the background level could be estimated.

The peak of the m.e.p.c. was found and the start and end were taken as points which exceeded the background mean level by 5% of the peak value. The background mean was then recalculated and by a reiterative process the duration of the m.e.p.c. and the level of the rest of the block were determined. The m.e.p.c.s often appeared to have a short plateau around the peak value. The 20–80% rise time and the rate of rise were determined from a linear regression fitted to the points between 5 and 95% of the peak value. The time constant of the fall was determined by fitting a regression line to the log values from 95 to 5% on the decay phase. For both rise and fall a correlation coefficient of 0·8 or better allowed inclusion of the m.e.p.c. in the series. In some instances the ratio of signal to noise was low and the exponential showed superimposed oscillations and a low correlation coefficient; in these cases a three-point smoothing technique was used and the analysis of the m.e.p.c. was repeated. The peak amplitude was reduced by less than 5% and the rise and fall times were not appreciably altered, but m.e.p.c.s excluded because of poor correlation coefficients could often now be included. The values determined by the two procedures were similar.

These analyses were carried out on a PDP 11/10 Digital computer with an analogue–digital converter (Laboratory Peripheral System) at St Mary's Hospital Computer Unit.

Drugs. The following drugs were used: acetylcholine chloride (British Drug Company, mol. wt. 182); carbamylcholine chloride (carbachol; Koch-Light Laboratories, mol. wt. 182·6); physostigmine sulphate (British Drug Company, mol. wt. 649); tetrodotoxin (Sankyo Co., mol. wt. 319).

Other statistical procedures. In the tables the median values are listed together with confidence limits of the median (approximately 95%: Colquhoun, 1971). Two-sample rank tests were also used (Goldstein, 1964).

RESULTS

Effect of temperature change upon membrane potential

Fig. 1 shows the effect upon membrane potential of a reduction in temperature of 15 °C which was recorded in a fresh preparation which had been isolated from the animal for 6 min. The muscle was set up in the recording chamber at 37 °C, and the membrane potential was recorded for 15–20 s to ensure a stable value. Readings were taken from several fibres, and the potential of the last cell in this initial series was observed for 1–2 min. The electrode was withdrawn, and the temperature was lowered to 22 °C by directing the flowing solution through a pre-cooled water-jacket. The temperature was lowered to 22 °C within 20–30 s, and the electrode was reinserted into the same cell. Fig. 1 shows that the membrane potential became depolarized by 5 mV and was stable. The potentials of several other cells were recorded at the lower temperature.

Attempts to record continuously the membrane potential during a sudden change of temperature of this magnitude were unsuccessful. The electrode was displaced during the manoeuvre, possibly due to a small movement of the muscle, and the procedure of withdrawal and reinsertion was adopted. Control experiments showed that the electrode could be reinserted without a change in potential. In four diaphragms the resting potential in fresh muscle in a solution with 4 mM-potassium was -85 mV at 37 °C (forty-one fibres; limits -84, -88 mV) and -79 mV at 22 °C (twenty fibres; limits -78, -82 mV; $P < 0{\cdot}01$).

Effect of temperature on miniature end-plate currents

Rise time. End-plates were localized and electrodes were inserted so that m.e.p.c.s could be recorded with membrane voltage-clamped at −80 mV at 37, 22 and 7 °C. Results were obtained from the same cell at different temperatures (Fig. 2), and the cooling interval varied between 30 s and 10 min. The results in Table 1 are from experiments in which there was a reduction of temperature, and also changes in the reverse direction. In seven end-plates readings were made at three temperatures and

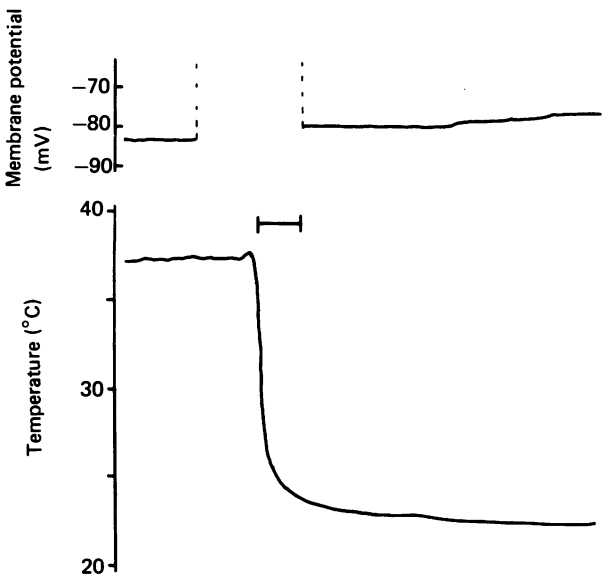

Fig. 1. Change of temperature (lower trace) and resting potential (upper trace). Initial resting potential −83 mV at 37 °C. The electrode was withdrawn, the temperature of the flowing solution was reduced to 22 °C, and the electrode was reinserted in the same end-plate. Horizontal scale: 20 s.

in other cases the effects at one or two temperatures were recorded. For results at any one temperature six to fifty m.e.p.c.s were collected and the amplitude, the 20–80 % rise time, and the time constant of the decay were estimated and averaged results obtained for each end-plate by the computer. In one cell at 7 °C only three m.e.p.c.s were recorded, and this result has been included in Table 1. The rise time found in this way was 162 μs at 37 °C (thirteen muscles; limits 104, 211 μs); at 22 °C the value was 564 μs (Table 1); and was much prolonged to 2·4 ms at 7 °C. The rate constant (Land *et al.* 1980) is 1·39/(rise time) and the temperature sensitivity is seen in the Arrhenius plot in Fig. 3 (upper regression line). The correlation coefficient is better than 0·9, and from the slope, which is $E/(2{\cdot}3\,R)$ where R the gas constant is 1·987 cal deg^{-1}, the apparent activation energy E is 14·4 kcal mol^{-1} deg^{-1}.

Fall. At 37 °C the time constant of decay of the m.e.p.c. was 324 μs (thirteen cells; limits 236, 386 μs). At 22 °C the value was prolonged to 1·73 ms (Table 1), and further cooling to 7 °C gave a value of 7·3 ms (nine cells; limits 5·0, 9·4 ms). The Arrhenius plot in Fig. 3 (lower regression) shows a steeper slope than that for the rise, with an apparent activation energy of 18·4 kcal mol^{-1} deg^{-1}.

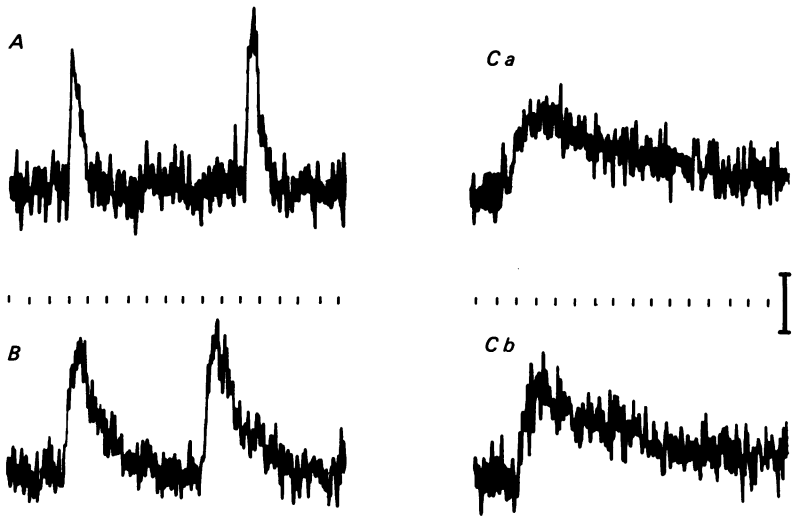

Fig. 2. Spontaneous miniature end-plate currents at −80 mV. *A*, 37 °C; *B*, 22 °C; *C a–b*, 7 °C; from the same fibre. A 10 kHz low-pass Gaussian filter was used Vertical scale: 2 nA. Time markings: 1 ms.

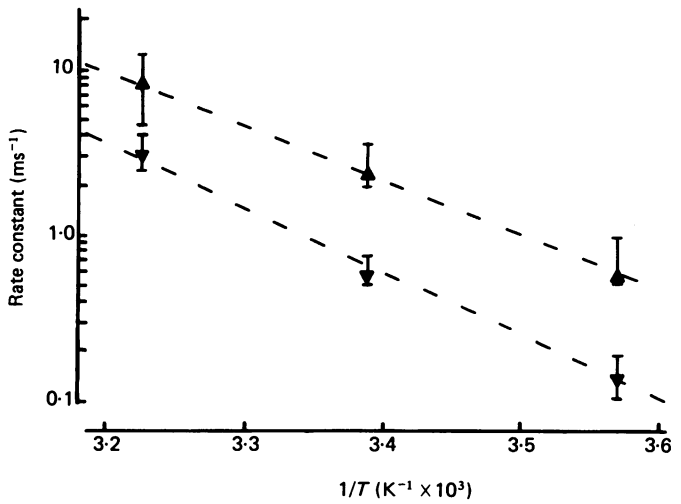

Fig. 3. Arrhenius plot. ▼, rate of fall of miniature end-plate currents, with ordinate as log rate and abscissa as inverse of absolute temperature. ▲, rate of rise, measured as 1·39/(20–80 % rise time). Points are medians from nine to thirteen end-plates, with 95 % limits, at 7, 22, 37 °C. Clamp voltage was −80 mV. The apparent activation energy from the slope of the regression is 18 kcal mol^{-1} deg^{-1} for the fall and 14 kcal mol^{-1} deg^{-1} for the rise.

TABLE 1. Miniature end-plate currents at different temperatures

	37 °C	22 °C	7 °C
Rise time (μs) (20–80 %)	162 (104, 211)	564 (371, 699)	2404 (1320, 2604)
n	13	10	9
Amplitude (nA)	4·6 (3·4, 6·7)	3·7 (2·3, 5·5)	1·5 (1·3, 1·65)
Time constant of decay (μs)	324 (236, 386)	1726 (1300, 1787)	7274 (4999, 9446)
Frequency (s^{-1})	3·0 (2·5, 4·6)	2·1 (1·6, 2·9)	0·4 (0·3, 0·8)

Medians are listed, with limits (95 %) in parentheses. n is number of end-plates.

Amplitude. At the lower temperatures the peak amplitude was reduced from that at 37 °C. (Table 1). However, the reduction in amplitude with temperature was not linear: there was only a small decrease in size from 37 to 22 °C, from a value of 4·6 nA to 3·7 nA. At 7 °C the magnitude of the m.e.p.c. was severely reduced to only just above the noise level, the median amplitude being 1·5 nA.

Frequency. There is great variation between cells in the frequency of occurrence of m.e.p.c.s even within the same preparation. However, in all cells where recordings were made at two or more temperatures, the frequency was reduced at the lower temperature. At 37 °C the median rate was 3·0 s^{-1} and at 22 °C it was 2·1 s^{-1} (Table 1). At 7 °C the rate was substantially reduced to 0·4 s^{-1}, or one m.e.p.c. every 2·5 s. This low frequency of occurrence at 7 °C unbalances the analysis, in that at this temperature fewer m.e.p.c.s were usually collected than at higher temperatures. The low frequency which was observed might be related to the low amplitude of m.e.p.c.s at 7 °C, because smaller m.e.p.c.s could not readily be distinguished from the background noise.

Extracellular recording of miniature end-plate potentials

Focally placed micro-electrodes when closely applied to the end-plate region record negative-going deflexions whose decay has a faster time course than internally recorded events (Fatt & Katz, 1952; Liley, 1956). The externally recorded miniature end-plate potential (inverted m.e.p.p.) can be considered as a measure of the end-plate current and does not require the voltage-clamp circuit. Fig. 4*C* shows an inverted m.e.p.p. obtained with a low-resistance electrode of 10 MΩ, with an internally recorded m.e.p.p. for comparison (*A*). Table 2 lists estimates of the rise time (20–80 %) obtained from inverted m.e.p.p.s recorded at 37 °C with the amplifiers having two different filter characteristics: 10 kHz low-pass Gaussian response (as used for internally clamped m.e.p.c.) and 20 kHz (Bessel response). The rise times estimated from the two methods are not dissimilar ($P > 0{\cdot}05$), although the rise is somewhat more rapid for the filter with the higher limit, with rise time 167 μs when filtered at 10 kHz and 139 μs for the 20 kHz filter. The values may be compared with the estimate of 162 μs from internally recorded m.e.p.c.s (Table 1).

Voltage sensitivity of miniature end-plate currents

Fig. 5 shows the time constant of decay recorded at clamp voltages between −60 and −150 mV. At all potentials and temperatures the decay of the m.e.p.c. appears as a single exponential, and the time constant of decay was greater with hyperpolarizing

voltages. The time constant is given by $T(0)$ exp $(-V/H)$, where $T(0)$ is the time constant at zero membrane voltage, V is clamp voltage and H is the hyperpolarization voltage which produces an e-fold increase in the time constant (Colquhoun, Large & Rang, 1977). At 22 °C the hyperpolarization factor was 122 mV six fibres; limits 75, 198 mV), which may be compared with the value of 109 mV found by Colquhoun *et al.* (1977). At 37 °C the relationship between the time constant of decay and

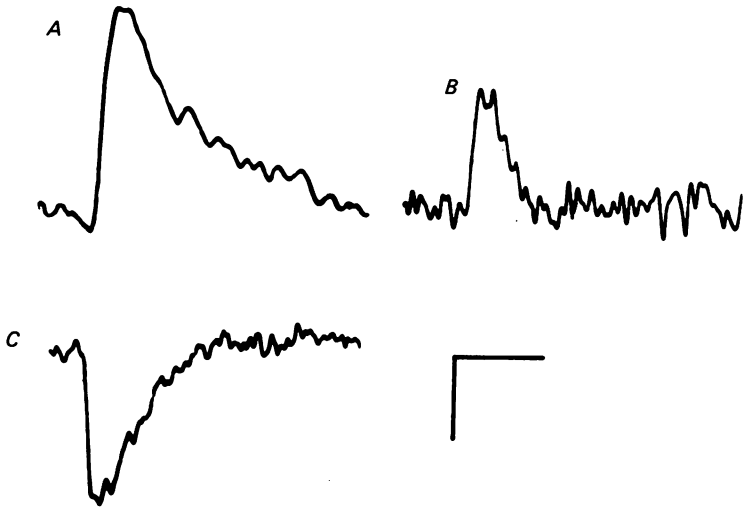

Fig. 4. *C*, inverted miniature end-plate potential recorded with an external electrode of resistance 10 MΩ. *A*, internally recorded miniature end-plate potential, for comparison. *B*, miniature end-plate current, with clamp at −80 mV. Vertical scale: for *A* and *C*, 0·2 mV; for *B*, 2 nA. Horizontal scale: 1 ms.

TABLE 2. Rise time of miniature end-plate potentials (μs, 20–80 %), recorded by external electrodes

Limiting filter	Rise time (μs)	*n*
10 kHz low-pass	167 (131, 226)	13
20 kHz low-pass	139 (102, 223)	6

Spontaneous potentials were recorded on the cathode ray oscilloscope (c.r.o.) and stored on the tape-recorder. In the first series frequency response was limited by the characteristics of the c.r.o. filter (10 kHz, Gaussian, −9 db per octave). In the second series the c.r.o. filter was set at 30 kHz and the response was limited by the filter in the tape-recorder (20 kHz, Bessel, −24 db per octave). Median values are shown, with limits in parentheses ($P > 0{\cdot}05$ by two-sample rank test). Temperature, 37 °C.

potential was flatter, which indicates less voltage sensitivity (Fig. 5, open circles) and the factor H had a value of 214 mV sixteen fibres; limits 153, 341 mV). The calculated time constant at zero membrane voltage $T(0)$ was 133 μs at 37 °C sixteen fibres; limits 99, 156 μs) while at 22 °C the value was 460 μs (limits 410, 550 μs).

The rise time of the m.e.p.c., estimated as the time for a 20–80 % rise, did not show voltage sensitivity in the range which was tested, there being no consistent effect as the membrane potential was changed (Fig. 5, triangles). In twelve cells hyperpolarization from −60 to −140 mV produced reduction and in nine other cells there was an increase in rise time.

The rate of rise calculated in nA s^{-1}, taken from the 20–80 % rise time and the amplitude in nA, was found to increase in all cells with hyperpolarization; it increased linearly, and closely followed the increase in amplitude of the m.e.p.c. as the cell was hyperpolarized.

Acetylcholine

Current produced by acetylcholine. The change in current produced by acetylcholine (10 μM) in the presence of tetrodotoxin (300 nM) and physostigmine (3 μM) was recorded. Solution which contained 10 μM-acetylcholine without the other drugs did not give any change in conductance, and the following procedure was adopted. An end-plate was clamped at −80 mV in solution containing tetrodotoxin and the

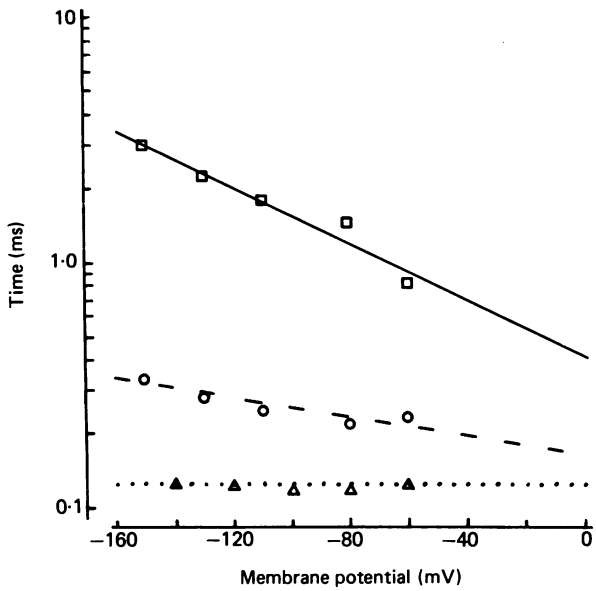

Fig. 5. Voltage sensitivity of the rise and fall of miniature end-plate currents. □, time constant of decay at 22 °C. ○, time constant of decay at 37 °C. △, rise time (20–80 %) at 37 °C.

spontaneous m.e.p.c.s were recorded for subsequent analysis. The solution was changed to one containing both tetrodotoxin and physostigmine and this solution was allowed to flow until the size of the spontaneous m.e.p.c. was clearly enhanced; after 3–5 min a record was obtained of background noise for subsequent spectral analysis. On changing to a solution which additionally contained acetylcholine there was a delay of 12–15 s attributed to the tubing dead space, and this was followed in most instances by a rapid increase in current within 20 s to a peak which varied between 29 and 96 nA in separate experiments (median 55 nA of 7) and which decayed to a plateau of current within about 1 min of the exposure of the tissue to the drugs (Fig. 6). During the increase in current the m.e.p.p.c.s disappeared. Occasionally there was a slow increase in current which reached a steady level without showing a peak. In those cells where continuous recording was possible the acetylcholine was left in contact for 4–7 min. Upon removal of acetylcholine the clamp current decreased rapidly so that in 1–3 min it had decayed to the original value. It was found that

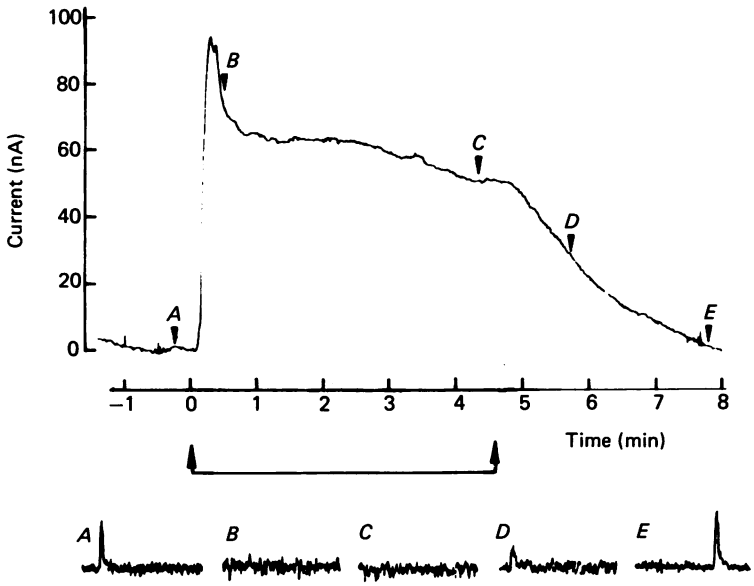

Fig. 6. Current induced by acetylcholine (10 μM) applied during time indicated by arrows, in presence of physostigmine (3 μM) and tetrodotoxin (300 nM). Temperature 37 °C; clamp at −80 mV. Inserts show high-gain trace before (*A*), during (*B*, *C*) and after (*D*, *E*) application. Miniature end-plate currents are seen in *A*, *D* and *E*: m.e.p.c.s were absent during application of acetylcholine. Time is measured from switch-over to solution containing acetylcholine.

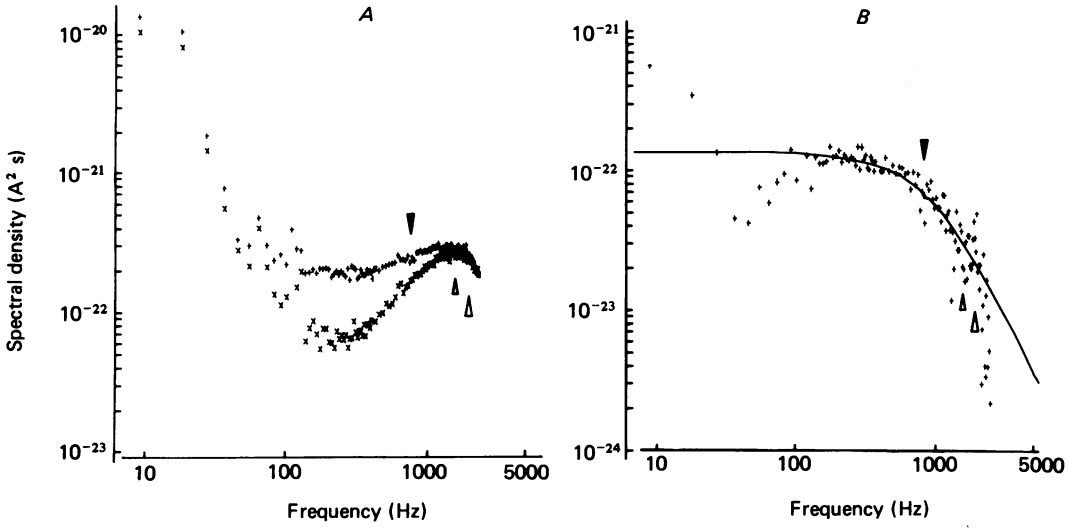

Fig. 7. *A*, power spectrum of current fluctuations, before application of acetylcholine (×) and during acetylcholine (10 μM) in presence of physostigmine (3 μM) and tetrodotoxin (300 nM) (+). *B*, spectrum of additional noise produced by acetylcholine, obtained by difference. The line which fits a single Lorentzian curve is shown. The filled pointer indicates the half-power frequency of 755 Hz. The open pointers indicate the cut-off due to the Butterworth filter (1 db and 3 db). Frequency (Hz) and spectral density (A^2 s) are on logarithmic scales.

m.e.p.c.s reappeared within 1 min of removal of acetylcholine at a time when the current had only partly recovered.

Fluctuation analysis. Fig. 6 shows the fluctuations at various intervals before, during and after application of acetylcholine at 37 °C. Before the drug the root mean square value of noise was between 180 and 280 pA, and was increased by acetylcholine by approximately 50 % (range 10–120 % in different experiments).

The spectral density function was calculated and plotted as in Fig. 7*A* as log spectral density against log frequency. The control spectrum was obtained from 200 blocks of recordings, free from m.e.p.c.s and other obvious artifacts. The control curve

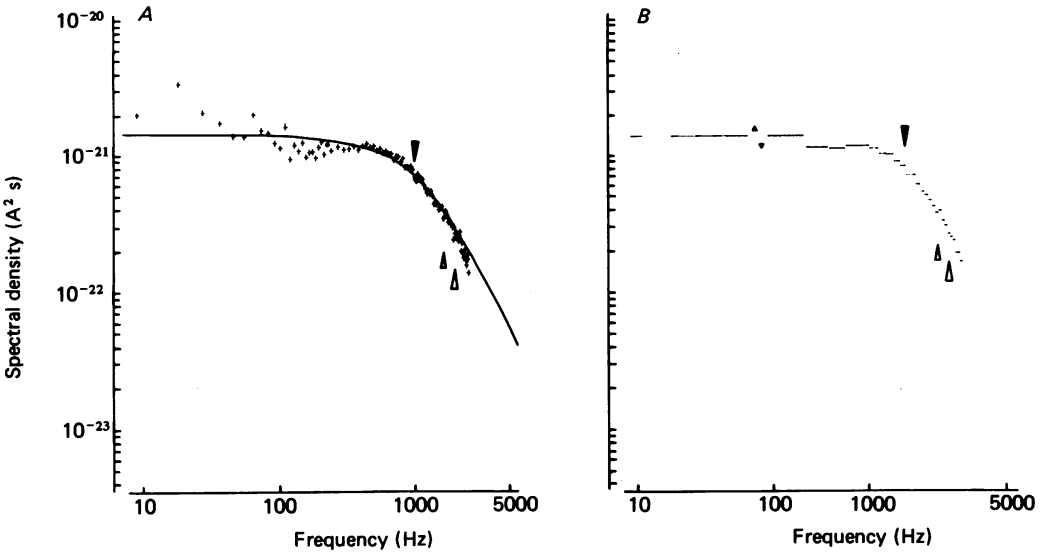

Fig. 8. Power spectrum of additional noise produced by acetylcholine. *A*, as in Fig. 7*B*, with line which shows the fit to a Lorentzian curve. *B*, plot derived from estimate of $S(0)$ obtained from median values of groups of points at low frequency and plotted by computer, as described in the text. Filled pointer shows half-power frequency at 800 Hz; open pointers show filter cut-off as in Fig. 7. Small filled pointers in *B* indicate 95 % limits of estimate of $S(0)$.

falls to a minimum, in this case at about 200 Hz, then rises to a maximum at 1·5 kHz, and then falls as the frequency approaches that of the cut-off produced by the filter (Butterworth 8 pole: see Methods). The curve at 37 °C resembles those at lower temperatures (Anderson & Stevens, 1973; Colquhoun *et al.* 1977).

Fig. 7*A* also shows the fluctuations in the presence of acetylcholine (10 μM) plus tetrodotoxin (300 nM) and physostigmine (3 μM). Fig. 7*B* shows the spectrum of the additional noise produced by acetylcholine, obtained by subtraction of the background noise. The net spectrum was fitted by a single Lorentzian curve of the form $S(0)/[1+(f/f_c)^2]$, where f_c is the half-power frequency, by methods which have been described above in which the term $S(0)$ and its limits are estimated from points on the 'plateau region' of the curve. Fig. 8*A* and *B* show a spectrum of acetylcholine noise at 37 °C from another experiment in which the curve was fitted by computer. In Fig. 7*B*, f_c is 755 Hz and in Fig. 8 the value is 800 Hz. Then with certain

reservations the mean channel lifetime from noise analysis τ can be obtained as $1/(2\pi f_c)$. In Table 3, τ is 237 μs (7; limits 170, 293 μs). The channel lifetime can also be obtained from the expression $S(0)/4\,var$, where *var* is the noise variance (Wray, 1981), and comes to 246 μs.

Single-channel conductance. From the spectral analysis and the gross current induced by acetylcholine the conductance of a single open channel can be found from the ratio var/i_m $(V-V_{eq})$, where *var* is the variance of the current fluctuations induced by acetylcholine, i_m is the mean induced current, V is the clamp potential

TABLE 3. Fluctuation analysis with acetycholine (10 μM)

		Time constant (ms)		Channel conductance (pS)			
	n	From f_c	From $S(0)$	From f_c	From $S(0)$	i (pA)	r.m.s.
22 °C	9	1·10 (0·91, 1·27)	1·03 (0·79, 1·21)	23 (13, 24)	15 (10, 18)	1·6 (0·9, 1·2)	+110%
37 °C	7	0·24 (0·17, 0·29)	0·25 (0·15, 0·29)	26 (8, 52)	25 (9, 47)	1·9 (0·6, 3·7)	+25%
37 °C external recording	4	0·26 (range 0·19, 0·31)	0·23	—	—	—	+30%

Acetylcholine (10 μM), physostigmine (3 μM) and tetrodotoxin (300 nM) were used. Estimates of the time constant τ and channel conductance were obtained from f_c the corner frequency, and also from $S(0)$ the spectral density at zero frequency. The single channel current i and the increase in root mean square current (r.m.s.) are shown. Some estimates by external recording at 37 °C are also included.

and V_{eq} is the reversal potential for acetylcholine which was taken as -10 mV. The channel conductance can also be obtained from the expression $S(0)/4i_m\tau$ $(V-V_{eq})$, where $S(0)$ is spectral density at low or zero frequency, and τ is the channel lifetime obtained from spectral analysis. These two methods yielded similar values of 26 and 25 pS for the channel conductance (Table 3). The current flowing through a single channel (Magleby & Weinstock, 1980) is given by var/i_m and the value of 1·9 pA is also shown in Table 3.

In most cases it was possible to compare the spectral density produced by acetylcholine during the initial period and also following application of the drug for several minutes (see Fig. 6). In two end-plates the estimated channel lifetime after 5 min became longer than the initial value, in two cases it became shorter and in others there was no change, so no consistent change was detected in this series. There was no significant change in channel conductance or single-channel current during the period of drug application.

Externally recorded noise. Recordings in the absence of acetylcholine were made with focally placed micro-electrodes. The cells were first impaled to locate the area where m.e.p.p.s of fast rise time and greatest amplitude were obtained, and the electrode was then withdrawn and was moved along the edge of the fibre until inverted m.e.p.p.s were recorded. The electrodes were of moderate resistance (10 MΩ) and produced a substantial degree of background noise.

Fig. 9 shows a trace recorded with an external electrode at 37 °C. Acetylcholine (5 μM) in the presence of tetrodotoxin (300 nM) and physostigmine (3 μM) produced a

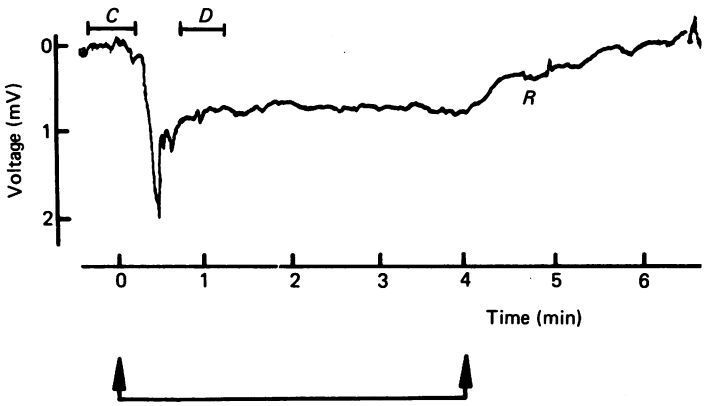

Fig. 9. Extracellular recording of voltage change produced by acetylcholine (5 μM) applied during arrows, in the presence of physostigmine and tetrodotoxin. Fluctuations recorded during the intervals *C* and *D*, before and after application of acetylcholine, were used to obtain the spectrum in Fig. 10. Miniature end-plate potentials (inverted) were present at *C*, disappeared during application of acetylcholine and reappeared with reduced amplitude at *R*. Trace retouched.

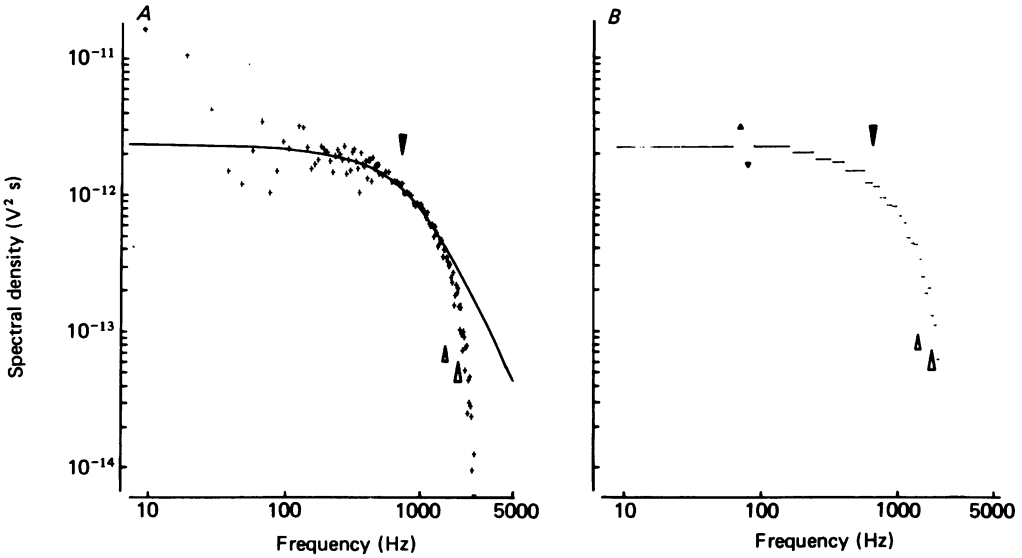

Fig. 10. *A*, spectrum of additional fluctuations produced by acetylcholine (5 μM) and recorded by external electrodes, as shown in Fig. 9. *B*, computer plot, as Fig. 8. Pointers as in Fig. 8. The half-power frequency is at 700 Hz.

peak voltage deflexion of 2 mV which rapidly decayed to a plateau, the level of which was maintained during the application. The form of the gross change was similar to that found with internal recording (Fig. 6). The m.e.p.p.s disappeared upon application and were restored within 1 min of withdrawal of acetylcholine and the potential returned to the original level. The noise increased in this case by 30 %. Fig. 10*A* gives the power spectrum of the additional noise caused by acetylcholine and in Fig. 10*B* the results have been plotted by computer. The half-power frequency was 700 Hz so that the estimated channel lifetime was 227 μs. In four experiments the channel lifetime from $S(0)$ was 226 μs (range 190, 302 μs), which is comparable to estimates obtained by internal recording (Table 3).

In Figs. 6 and 9 there was an initial peak in the record which rapidly decayed to a lower value. This pattern was seen in four out of seven traces made with internal electrodes, and in all traces made with external electrodes, when the recording was known to be focal as shown by inverted m.e.p.p.s. In eight records (four internal and four external) the 'plateau' was 55 % of the peak value (range 33, 60 %).

A similar peak was seen if the increase in the variance induced by acetylcholine was plotted against time. The ratio of peak to early 'plateau' level was 59 % (8; range 37, 77 %).

Carbachol

The additional current produced by carbachol (40 μM) was recorded both by voltage clamp and by focally placed external electrodes, and in both instances (Fig. 11) the current showed an initial peak which decayed to a 'plateau' and was restored to the initial value only on withdrawal of the drug. Spontaneous m.e.p.c (or m.e.p.p.s) were not seen during application of the drug, as with acetylcholine.

Fig. 12*A* and *B* show the power spectrum of the additional noise from a cell clamped at -80 mV at 37 °C, and Fig. 12*C* and *D* are plots of results from external recording. Single Lorentzian curves have been fitted, and from three internal and three external recordings (Table 4) the channel lifetime from $S(0)$ is 83 μs (6; limits 56, 102 μs).

All six records indicated an initial peak of current which rapidly fell to a lower value (Fig. 11). The level of the early 'plateau' was 60 % of the peak (6; range 43, 74 %).

The ratio of channel open lifetime obtained with carbachol as compared with that with acetylcholine was 83/237 or 0·35 at 37 °C. This is similar to that found by Katz & Miledi (1972) and Colquhoun *et al.* (1977).

DISCUSSION

The high site density of receptors in the post-junctional membrane has led to the concept that a single quantum of transmitter will produce a saturating concentration over a small area (Matthews-Bellinger & Salpeter, 1978). The rise time of a miniature end-plate current is correlated with the amplitude (Negrete, Del Castillo, Escobar & Yankelevich, 1972) and the rise is slowed when the receptor site density is decreased by α-bungarotoxin (Land, *et al.* 1980). The contribution to the rise time produced by diffusion, binding and isomerization or other chemical activation is uncertain (Adams, 1980; Land *et al.* 1980; Dwyer, 1981). In rat muscle the temperature sensitivity of the rise has been found to be moderately high with apparent activation energy 14 kcal mol^{-1} deg^{-1} from 7 to 37 °C, so that the contribution of diffusion to the process is unlikely to be a major factor and the rising phase of the miniature end-plate current may be largely attributed to non-diffusional events which include

binding and isomerization. The rise showed no sensitivity to voltage change in rat muscle at 37 °C over the range which was tested, and this contrasts with Dwyer's findings in the frog (1981) but resembles results in other studies (e.g. Gage & McBurney, 1975). The rise time was similar when external or internal recording was employed (Tables 1 and 3) and the frequency limitations of the clamp circuit used

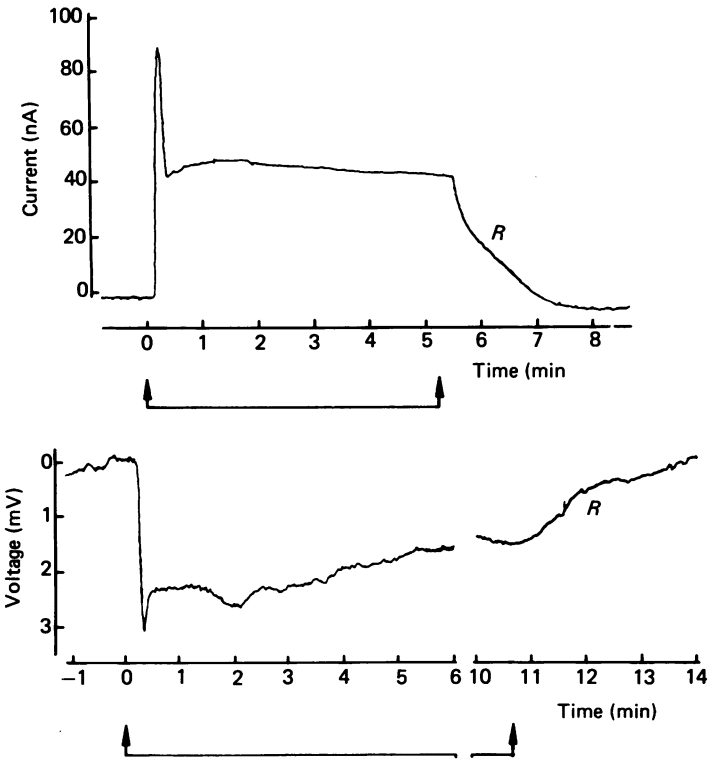

Fig. 11. Carbachol (40 μM) applied between arrows. Upper trace shows internal recording of current at 37 °C and −80 mV. Lower trace shows external recording of voltage change. *R* indicates reappearance of miniature end-plate current (upper trace) and of inverted miniature end-plate potentials (lower trace).

for internal recording did not appear to introduce serious distortion. The falling phase of the miniature end-plate current (Table 1) was sensitive to voltage although the sensitivity was less at body temperature than at 22 °C. External records of the falling phase have not been used, as the method is known to introduce an artifact which prolongs the apparent decay (Katz & Miledi, 1973).

The power spectra of the fluctuations produced by acetylcholine and by carbachol have been fitted to single Lorentzian curves, and there was no trace of double curves as found for skeletal muscle by Dionne & Parsons (1981) and Dreyer, Walther & Peper (1976*b*). A method of curve fitting has been adopted in which $S(0)$, the spectral density for low or zero frequency is constrained to be within the limits of the values found at the 'plateau' region at low frequencies, and this has allowed computer fitting of the results (Fig. 8, 10 and 12), which compares well with other methods. From the fitted curve the open lifetime of a channel can be found if certain assumptions which

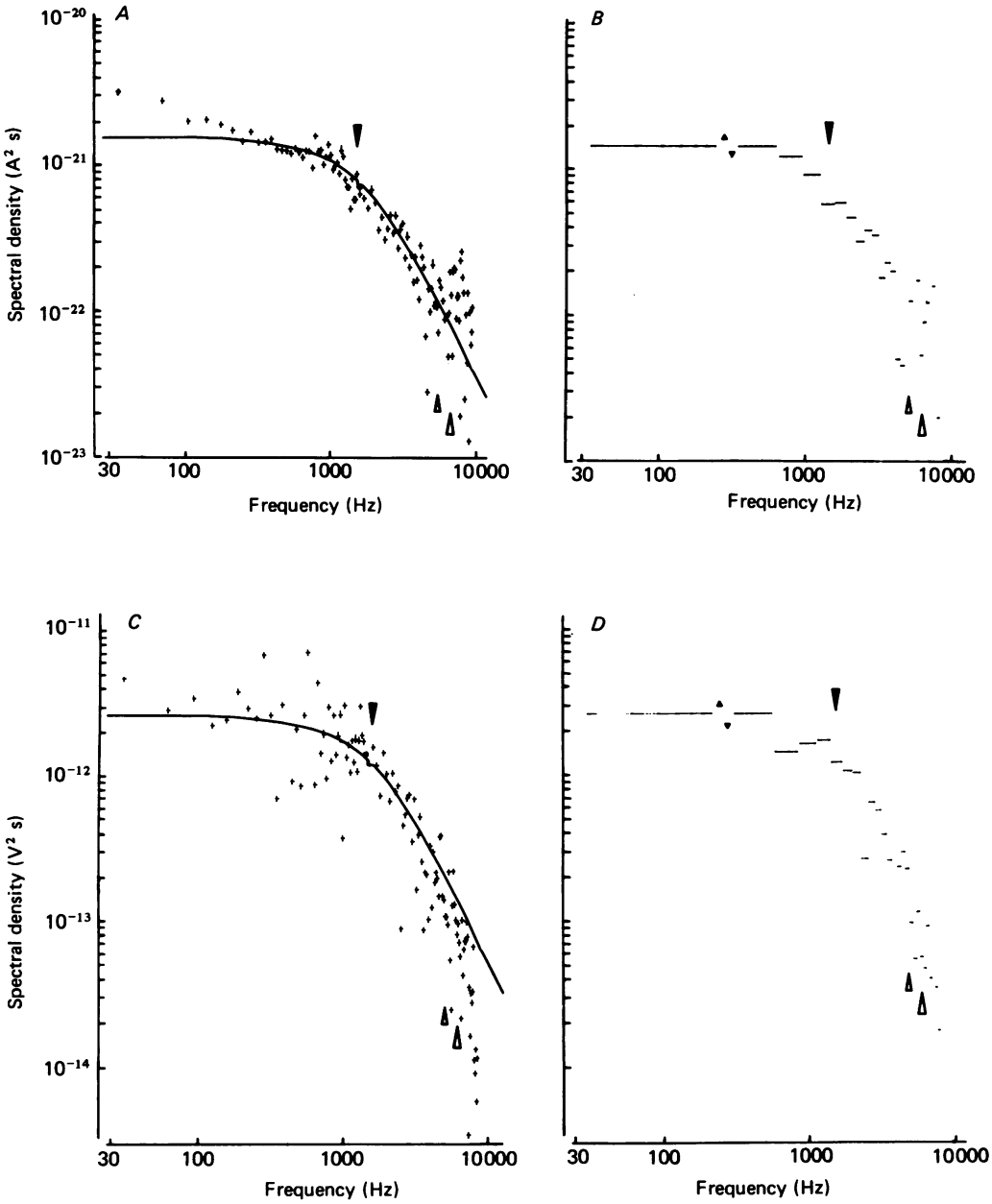

Fig. 12. Carbachol (40 μM) at 37 °C. *A* and *B*, spectrum of additional fluctuations obtained by internal recording at −80 mV, as Fig. 8. Half-power frequency 1·4 kHz. *C* and *D*, spectrum obtained by external recording, as Fig. 10. Half-power frequency 1·5 kHz.

have been listed by Colquhoun *et al.* (1977) are valid. The estimated mean lifetime at 37 °C for channels opened by acetylcholine is 237 μs and may be compared with the value of 0·3 ms in mouse muscle at 39 °C (Dreyer, Müller, Peper & Sterz, 1976*a*). The time constant of the falling phase of the miniature end-plate current was consistently longer at 37 °C then the open time obtained from noise analysis. Similar behaviour has been found in rat muscle at 20 °C (Colquhoun *et al.* 1977) and the discrepancy may represent delayed clearance of transmitter from the cleft (Colquhoun *et al.* 1977).

TABLE 4. Fluctuation analysis with carbachol (40 μM) at 37 °C

	Time constant (μs)		Channel conductance (pS)			
	From f_c	From $S(0)$	From f_c	From $S(0)$	i (pA)	r.m.s.
Internal	65	75	17	20	1·2	+5%
	114	90	17	14	1·2	+9%
	110	102	15	14	1·0	+6%
External	74	68	—	—	—	+3%
	49	56	—	—	—	+12%
	114	90	—	—	—	+4%

Successful recordings were made from six end-plates (three with internal, and three with external electrodes). Median value from $S(0)$ is 83 μs (6).

With peak amplitude of 4·6 nA for the miniature end-plate current (Table 1) and 1·9 pA for the single-channel current (Table 3), the number of channels open at the peak is 2300 at 37 °C, and a similar calculation gives 2400 channels at 22 °C. The values may be compared with 1700 channels at 8 °C in frog muscle (Anderson & Stevens, 1973) and 1300 in frog at 15 °C (Magleby & Weinstock, 1980). With continued application of acetylcholine a current of 50 nA would indicate that at any one time $2{\cdot}6 \times 10^4$ channels are open, which compares with figures of 5×10^4 (Neher & Sakmann, 1976) and $2{\cdot}1 \times 10^4$ (Colquhoun, 1981). If the number of sites in rat muscle is $4{\cdot}7 \times 10^7$ (Miledi & Potter, 1971) then the fraction of channels open at any one time would be 0·06%, which is similar to calculations made in frog muscle (Anderson & Stevens, 1973). With a channel open lifetime of 237 μs the frequency of channel opening (Wray, 1981) is $(2{\cdot}62 \times 10^4)/(237 \times 10^{-6})$ or 11×10^7 s^{-1}, which is smaller than the value of 39×10^7 s^{-1} obtained for acetylcholine in cat muscle (Wray, 1981).

In rat muscle at 37 °C acetylcholine and carbachol produced an initial peak of current, which was followed by a rapid fall to level which then declined slowly and appeared to be stable. This effect was not found in all cases but was always present when focal recording was confirmed by the presence of inverted miniature end-plate potentials. A similar effect has recently been shown in frog muscle (Feltz & Trautmann, 1982), in which the 'fade' or desensitization is biphasic with a rapid effect which is followed by a slower action. The appearance of a peak may be associated with high flow-rate, which was 40–45 ml min^{-1} in rat muscle and 15 ml min^{-1} for frog (Feltz & Trautmann, 1982). In membrane vesicles from the electric organ of *Torpedo*, carbachol produces a biphasic desensitization (Neubig & Cohen, 1980), and similar results have been obtained by Hess, Cash & Aoshima (1979) who worked with

Electrophorus. In the present study an initial peak and decline to a lower level were also found when the variance of the additional fluctuations was estimated. The ratio of peak values to these in the early plateau was similar with current and variance, and hence there was no indication of a change in the current through a single channel. The apparent lifetime of a channel also showed no consistent change with time, and these negative findings are in agreement with those of Anderson & Stevens (1973) who found that desensitization produced a fall in total current but caused no change in channel conductance or channel lifetime in frog muscle. This would accord with the view that the changes responsible for early desensitization produce a fall in the frequency of channel opening without significant alteration in the current which is passed during a single opening. A similar interpretation has been made by Wray (1981).

The use of the 'patch-clamp' method for single channel recording in frog muscle has shown a succession of rectangular currents (Neher & Sakmann, 1976). With high resolution even at low agonist concentration the current pulses are found to be interrupted by brief gaps (Colquhoun & Sakmann, 1981). It is suggested that the noise spectrum would consist of only a single component, and the time constant derived from the Lorentzian curve might correspond to the mean length of a burst of pulses.

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